

BACKGROUND OF THE INVENTION

C1 1. Field of the Invention

On page 3, before line 6, insert the following heading:

C2 2. Description of the Related Art

On page 4, before line 18, insert the following heading:

C3 SUMMARY OF THE INVENTION

On page 5, before line 20, insert the following headings and paragraphs:

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a protein sequence of wild-type staphylokinase, SakSTAR (SEQ ID NO: 10). Numbering starts with the NH₂-terminal amino acid of mature full length staphylokinase.

C4 Fig. 2 is a time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (open circles, n=9), SakSTAR (K74A) (closed circles, n=11) or SakSTAR (K74A,E75A,R77A) (open squares, n=6) in patients with peripheral arterial occlusion. The data represent median values and interquartile ranges, in $\mu\text{g/ml}$.

Fig. 3 is a protein sequence of wild-type staphylokinase, SakSTAR with indicated amino acid substitutions.

Squares: single amino acid substitutions; circles: combined (2 to 3) amino acid to Ala substitutions.

Fig. 4 shows temperature stability of SakSTAR, (A); SakSTAR (K74Q, E80A, D82A, K130T, K135R), (B); SakSTAR (E65D, K74R, E80A, D82A, K130T, K135R), (C); and SakSTAR (K35A, E65D, K74Q, E80A, D82A, K130T, K135R), (D).

(○): 4°C; (●): 20°C; (▽): 37°C; (▼): 56°C; (□): 70°C.

C4 Fig. 5 is a time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (circles, n=15), SakStar (K74Q, E80A, D82A, K130T, K135R) (squares, n=6) or SakSTAR (E65D, K74R, E80A, D82A, K130T, K135R) (triangles, n=6) in patients with peripheral arterial occlusion. The data represent median values and 15-85 percentile ranges, in µg/mL.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

On page 9, please delete the first complete paragraph and insert the following replacement paragraph:

C5 The plasmids encoding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated *sakSTAR* variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the *staphylokinase* gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' (SEQ ID NO: 1) to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the *staphylokinase* gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3' (SEQ ID NO: 2).

On page 19, please delete the first complete paragraph and insert the following replacement paragraph:

C6 The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the *pMEX.SakSTAR* vector as template, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC (SEQ ID NO: 3), which destroys the unique *Hind*III site located 3' to the staphylokinase encoding gene in *pMEX.SakSTAR* and allows to counter-select the non-mutant progeny by *Hind*III digestion. The deletion of the *Hind*III site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the *pMEX.SakSTAR* plasmid using the primer 818A located at the 5' end of the *sakSTAR* gene (5' CAGGAAACAGAATTCAGGAG) (SEQ ID NO: 1) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACCTTGG) (SEQ ID NO: 4). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with *Eco*RI and *Pst*I, and ligated into the corresponding sites of *pMEXSakSTAR*.

On page 20, please delete the first complete paragraph and insert the following replacement paragraph:

C7 The plasmids encoding all the other variants listed in Table 3 were constructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR)(24) using *pMEX.SakSTAR* or available plasmids encoding SakSTAR variants as template. Two

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C7 fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *SakSTAR* coding region.

On page 32, please delete the first complete paragraph and insert the following replacement paragraph:

C8 The variants *SakSTAR*(K102C) and *SakSTAR*(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using *pMEX.SakSTAR* encoding *SakSTAR* as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (for the construction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) (SEQ ID NO: 6) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward) (SEQ ID NO: 7) for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) (SEQ ID NO: 8) and TAG GGA

fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *SakSTAR* coding region.

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The variants *SakSTAR*(K102C) and *SakSTAR*(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using *pMEX.SakSTAR* encoding *SakSTAR* as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (for the construction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) (SEQ ID NO: 6) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward) (SEQ ID NO: 7) for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) (SEQ ID NO: 8) and TAG GGA